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Case Report—

Data from 11 Years of Molecular Typing Infectious Bronchitis Virus Field Isolates

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SUMMARY. In 1993, a new molecular typing method for infectious bronchitis virus (IBV) was introduced. This method uses reverse transcriptase-polymerase chain reaction (RT-PCR) and restriction fragment length polymorphism (RFLP) analysis of the spike gene to obtain RFLP patterns that correlate with serotype. Using that test at the Poultry Diagnostic and Research Center (PDRC, University of Georgia, Athens, GA), we have identified a total of 1523 IBV isolates in the past 11 yr. The data were obtained from clinical samples submitted to our laboratory from birds with clinical signs characteristic of IBV infection. The samples are primarily from the southeastern United States but are also from many other states as well as from outside the United States. Most of the isolations occurred during July, followed by May, April, November, October, and January. The fewest number of isolates identified on an annual basis was 20 in 2003. An unusually high number of isolations occurred in 1997 (318 isolations) and 1999 (246 isolations), which coincided with the GAV variant virus and GA98 variant virus outbreaks respectively. By far, the Ark-DPI strain was the most frequently identified type of IBV and ranged from 23% to 65% of total isolations per year. Ark-like isolates, defined as having a similar but unique RFLP pattern from the Ark-DPI vaccine strain were identified every year of the study except in 1996. In addition, new Ark-like isolates continued to emerge each year (except in the year 2000) beginning in 1997, reflecting the ability of that IBV type to undergo genetic drift. Eighty-two different variant viruses were identified although only two (GAV and GA98) became persistent and caused widespread disease. Some viruses tended to be geographically restricted to a given area (CAV in California and MX97-8147 in Mexico), whereas others were widespread (Ark-DPI, Conn, DE072, and Mass). The Florida, Gray, Holte, Iowa, and JMK types were not detected during the 11-yr period, and no foreign virus types were detected in the United States. These data show that IBV variant viruses are consistently circulating in commercial poultry and are capable of causing disease outbreaks. Our observations highlight the importance of constantly monitoring IBV as well as other coronaviruses like severe acute respiratory syndrome-coronavirus that have the ability to change and emerge to cause disease in a susceptible host.

RESUMEN. *Reporte de Caso*—Datos obtenidos durante 11 años de tipificación molecular de aislamientos de campo del virus de bronquitis infecciosa.

En 1993 se introdujo un nuevo método molecular para tipificar el virus de bronquitis infecciosa. Este método emplea la prueba de la transcriptasa reversa-reacción en cadena por la polimerasa y el análisis de la longitud de los fragmentos de restricción del gen S1 para obtener un patrón de fragmentos que se correlaciona con el serotipo. Mediante la aplicación de dicha prueba en el Centro de Diagnóstico e Investigación Avícola de la Universidad de Georgia durante los últimos 11 años, hemos identificado un total de 1523 aislamientos del virus de bronquitis infecciosa. Los aislamientos fueron obtenidos a partir de muestras clínicas de aves con signos clínicos característicos de la infección por el virus de bronquitis infecciosa enviadas a nuestro laboratorio. Las muestras procedieron principalmente del Sureste de los Estados Unidos, sin embargo, se obtuvieron muestras provenientes de otros estados de los Estados Unidos y de otros países. La mayoría de los virus fueron aislados durante el mes de Julio, seguido por Mayo, Abril, Noviembre, Octubre y Enero. El menor número de virus aislados por año fue de 20 aislamientos durante el año 2003, mientras que el mayor número de virus aislados correspondió a 318 en 1997, y de 246 en 1999, coincidiendo con las epidemias ocasionadas por los virus variantes de Georgia GAV y GA98, respectivamente. La cepa Arkansas-DPI fue la cepa del virus de bronquitis infecciosa identificada con mayor frecuencia, correspondiendo del 23% al 65% del total de aislamientos por año. En cada año, con excepción del año 1996, se identificaron aislamientos similares al tipo Arkansas, caracterizados por presentar un patrón único en la longitud de sus fragmentos de restricción similar al observado con la cepa vacunal Arkansas DPI. Además, en cada año a partir del año 1997, con excepción del año 2000, se observó una aparición continua de nuevos aislamientos similares al tipo Arkansas, reflejando la capacidad de esta cepa de generar mutaciones antigénicas mayores. Se identificaron un total de 82 cepas variantes diferentes, aunque únicamente dos (GAV y GA98) llegaron a persistir y ocasionaron la diseminación de la enfermedad. Se observó la tendencia de algunos virus de permanecer restringidos a ciertas zonas geográficas (CAV en California y MX97-8147 en México), mientras que otros virus se diseminaron ampliamente (Ark-DPI, Conn, DE072 y Mass). Durante los 11 años de este estudio no se detectaron los virus Florida, Gray, Holte, Iowa y JMK y no se detectó la presencia de virus de bronquitis infecciosa de otros países en los Estados Unidos. Los datos muestran que los virus variantes de bronquitis infecciosa se encuentran circulando constantemente en la avicultura comercial y que son capaces de ocasionar brotes de la enfermedad. Nuestras observaciones resaltan la importancia de una constante evaluación del virus de bronquitis infecciosa, al igual que otros coronavirus como el coronavirus

causante del síndrome respiratorio agudo severo, el cual tiene la capacidad de cambiar y emerger, causando la enfermedad en huéspedes susceptibles.

Key words: IBV, molecular typing, RT-PCR, RFLP, incidence, distribution, variant, disease outbreaks, emerging strains

Abbreviations: Ark = Arkansas; Ark-DPI = Arkansas-Delmarva Poultry Industry; CAL99 = California 99; CAV = California variant; Conn = Connecticut; DE072 = Delaware 072; GA98 = Georgia 98; GAV = Georgia variant; IBV = infectious bronchitis virus; Mass = Massachusetts; MX = Mexican; NE95 = Nebraska 95; PDRC = Poultry Diagnostic and Research Center; RFLP = restriction fragment length polymorphism; RT-PCR = reverse transcriptase-polymerase chain reaction; SARS-CoV = severe acute respiratory syndrome-coronavirus

Infectious bronchitis virus (IBV) is a group III coronavirus that causes a highly contagious upper-respiratory tract disease in chickens (3). Different serotypes of the virus have been identified using virus neutralization testing in embryonating eggs (6), and modified live vaccines have been developed to control the disease. Generally, different serotypes do not cross-protect. Therefore, the serotype of the virus causing the disease must first be determined so that the birds can be properly vaccinated.

In 1993, a new molecular typing method for IBV was developed, which changed the way the virus is identified (8,9). That method uses the reverse transcriptase-polymerase chain reaction (RT-PCR) and restriction fragment length polymorphism (RFLP) analysis of the spike gene to obtain RFLP patterns that correlate with serotype (2,8,9). The test is rapid and has led to the identification of a tremendous number of virus isolates, which was not possible with the traditional virus-neutralization test in embryonating eggs.

Ongoing typing of IBV isolates is important so that new potentially problematic viruses can be detected early and controlled. Examining virus types over time is also important for understanding epidemiological aspects and evolutionary trends in IBV and in coronaviruses in general (7). Herein, we summarize the number, origin, and type of IBV isolates received at the Poultry Diagnostic and Research Center (PDRC, University of Georgia, Athens, GA) from 1994 through 2004. Molecular typing data for coronaviruses over an extended period of time have not been previously reported, making this information unique and valuable because it provides perspective on the incidence and distribution of IBV that can be used to better understand the emergence of new infectious bronchitis viruses as well as coronaviruses causing diseases in other animals and humans.

MATERIALS AND METHODS

Clinical samples. The samples used in this study were from birds with clinical signs consistent with infectious bronchitis submitted as diagnostic cases to the PDRC diagnostic laboratory. All clinical samples positive for IBV were molecularly typed. The samples were inoculated into the allantoic cavity of 9–11-day-old embryonating eggs and allantoic fluid was harvested 48 hr postinoculation. The samples were passaged in eggs a maximum of three times. Following each passage, the allantoic fluid was used for viral RNA extraction and tested for the presence of IBV nucleic acid by RT-PCR. If IBV was detected, the virus was typed and no further passages were conducted.

Isolates of IBV were identified from cases submitted to the PDRC diagnostic laboratory over an 11-year period from July 1994 to December 2004. Isolates were from the Southeastern region of the United States (Alabama, Arkansas, Georgia, Kentucky, Mississippi, North Carolina, South Carolina, Tennessee, Virginia) as well as from California, Connecticut, Delaware, Florida, Iowa, Illinois, Kansas, Maryland, Michigan, Minnesota, Missouri, New Jersey, New York, Nebraska, Ohio, Oklahoma, Pennsylvania, and Texas. Foreign isolates were obtained from Canada, Chile, Mexico, Peru, Israel, and Saudi Arabia. All isolates from foreign countries were first inactivated with an equal volume of buffered (pH 4.5) phenol (AMRESCO, Solon, OH), then shipped to our laboratory using import permit number 42290, U.S. Department of Agriculture, Animal and Plant Health Inspection Service (Veterinary Services, Riverdale, MD).

Viral RNA extraction and RT-PCR. Viral RNA extraction and RT-PCR/RFLP were conducted as previously described (8). Viral RNA was purified from allantoic fluid using the High Pure RNA extraction kit (Roche Diagnostics Corporation, Indianapolis, IN). The RT-PCR was conducted with the Titan One Step kit (Roche Diagnostics Corporation). The primers for the RT-PCR amplify a 1750-base pair fragment containing the entire S1 subunit of the spike gene. The name and sequences of the original RT-PCR primers were S1OLIGO5′, forward primer 5′ TGAAAACTGAACAAAAGACA3′ and S1OLIGO3′ reverse primer 5′CATAACTAACATAAGGGCAA3′ (9). In 1997, the forward primer was redesigned in response to changes in the virus. The new primer was designated NEWS1OLIGO5′ and the sequence was 5′TGAAACTGA ACAAAAGAC3′ (8). In 2000, the reverse primer was redesigned in response to changes in the virus and designated Degenerate 3′ 5′ CCATAAGTAACATAAGGRCRA3′ (10). A universal set of primers MIBVPCR 3′TAAGCTTTCAGTGGCTTGCTAAGTGTGAACC5′ and NIBVPCR 3′TGGATCCACCGCTACCTTCAAACTTGGGCGG5′, complementary to conserved regions of the genome, amplify a 1020-base sequence in all strains of IBV tested to date and were used as previously reported (1) to confirm the presence of IBV in samples that were negative with the S1 primers.

The S1 amplicons were gel purified on a 1% agarose gel using GenElute spin columns (Sigma-Aldrich Co., St. Louis, MO) and Microcon 30 columns (Millipore, Bedford, MA) per the manufacturer's instructions and then used for the RFLP analysis. Restriction enzymes *Bst*YI, *Hae*III, and *Xcm*I (New England Biolabs, Inc., Beverly, MA) were used in separate reactions according to the manufacturer's instructions to digest the amplicons, and the resulting DNA fragments were separated on a 1% agarose gel and visualized with EtBr staining and an ultraviolet light transilluminator.

RESULTS

A total of 1511 IBV isolates over an 11-yr period were typed. Two additional isolates could not be typed because the S1 gene was not amplifiable, although both were determined to be IBV based on amplification of a highly conserved region in the genome (MIBVPCR and NIBVPCR primers). Most IBV isolations occurred in July followed by May, April, November, October, and January (Fig. 1). The fewest number of isolates identified on an annual basis was 20 in 2003. The most isolates (318) were typed in 1997 (Fig. 1), which coincided with an outbreak of a new variant virus designated Georgia Variant (GAV). The majority of the GAV isolates were identified in May, followed by July and April. An unusually high number of isolations (246) also occurred in 1999 (Fig. 1), which coincided with the identification of another new variant virus designated Georgia 98 (GA98) (11). Most isolations of GA98 were in July, followed by June and May.

The Arkansas-Delmarva Poultry Industry (Ark-DPI) strain was the most frequently identified IBV type. It was calculated to be 42.4% of total IBV isolates (Fig. 2), with isolations ranging from 23% in 1997 to 65% in 2003. The next most common IBV types identified were Connecticut (Conn), 13.4% of total isolations, and

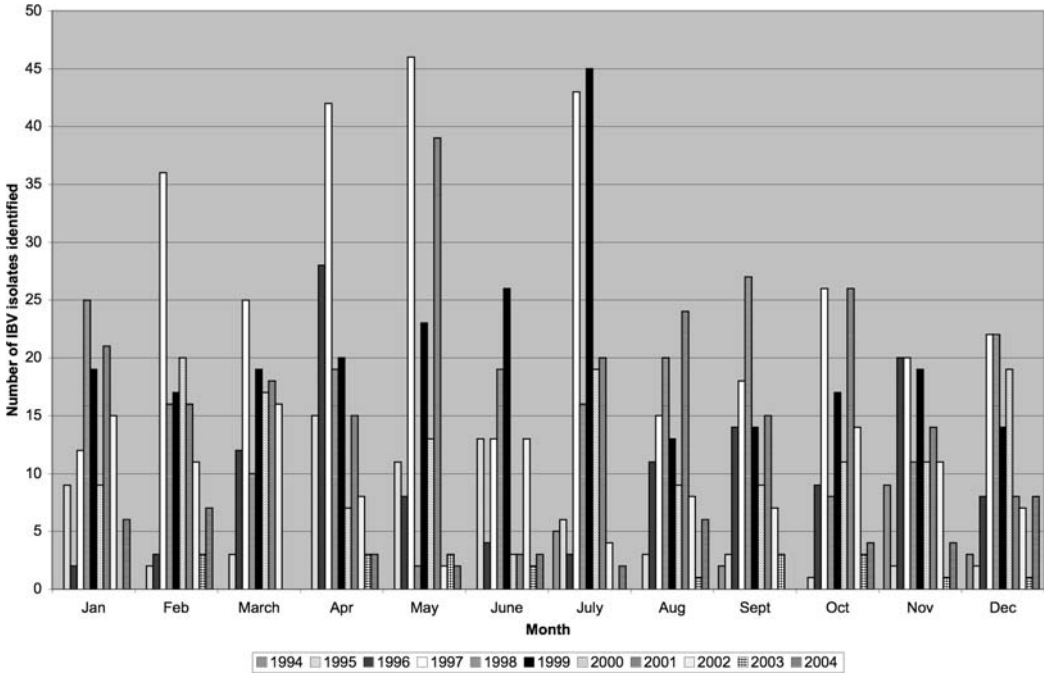

Fig. 1. Number of IBV isolates identified at PDRC each month from July 1994 to December 2004.

Massachusetts (Mass), 10.2% of total isolations. Rarely (less than 3% of cases) were mixed infections of two or more IBV types identified in the same sample. Over the 11-yr period, we detected variant IBV types 118 times, or 7.8% of total IBV isolates, and 82 different variant viruses were identified.

Ark-like viruses were first observed in 1995 and have been isolated every year since 1997 (Fig. 3). The RFLP patterns for six of the most frequently isolated Ark-like viruses are presented in Fig. 4. All together, 23 different Ark-like RFLP patterns have been observed in Arkansas, Delaware, Georgia, Kentucky, Maryland, Missouri, Mississippi, North Carolina, Oklahoma, Pennsylvania, South Carolina, and Texas.

Examining the origin of IBV types shows that vaccine type viruses (Ark, Conn, DE072, and Mass) are widespread, occurring throughout the southeastern United States and in most of the Midwest and Western states. Only Mass-type viruses and variant viruses were detected outside the United States, except for one isolation of Conn made in Chili. Some virus types were restricted to a given geographic area. The California variant (CAV) isolate was only isolated in California. Likewise the Mexico (MX) 97-8147 isolate was only isolated in Mexico (5). No foreign virus types (2) were detected in the United States. Finally, it is interesting to note that some of the most highly characterized serotypes of IBV, namely Florida, Gray, Holte, Iowa, and JMK, were not isolated during the 11-yr period.

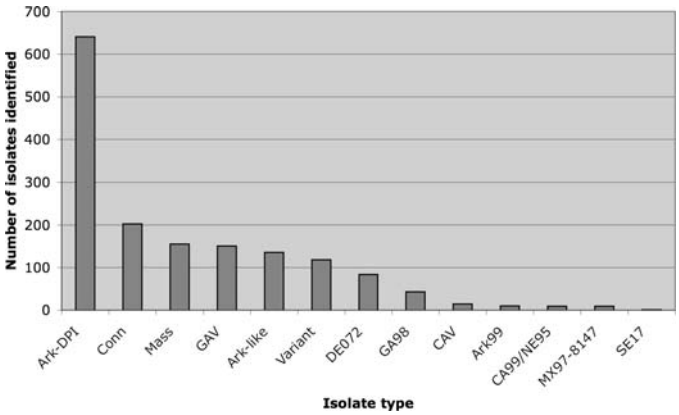

Fig. 2. Number of each IBV type identified at PDRC over an 11-yr period. Ark-DPI = Arkansas-Delmarva Poultry Industry, Mass = Massachusetts, Conn = Connecticut, DE072 = Delaware 072, GA98 = Georgia 98, Ark-like = Arkansas-like, Ark99 = Arkansas 99, GAV = Georgia variant, CAV = California variant, CA99/NE95 = California 99/Nebraska 95, variant = any virus with a unique RFLP pattern, MX97-8147 = Mexico 97-8147, SE17 = Southeast 17.

DISCUSSION

In this study, the RT-PCR/RFLP test was used to type 1511 isolates of IBV over an 11-yr period. The greatest number of IBV isolates was obtained during the month of July, followed by May and April. It is interesting that most IBV isolates, including the GAV and GA98

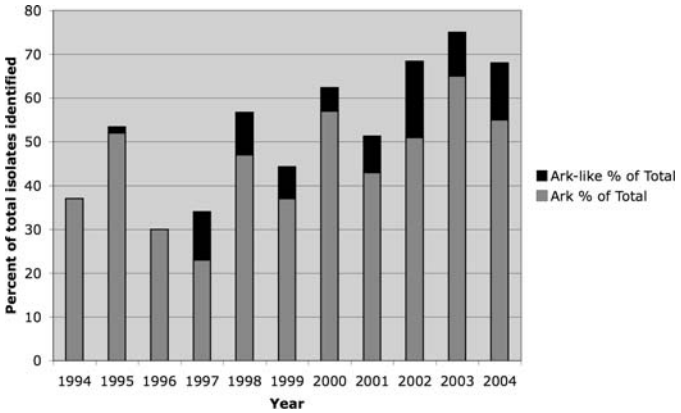

Fig. 3. Percent of Arkansas (Ark) and Arkansas-like (Ark-like) virus types identified compared with total IBV isolates identified by year.

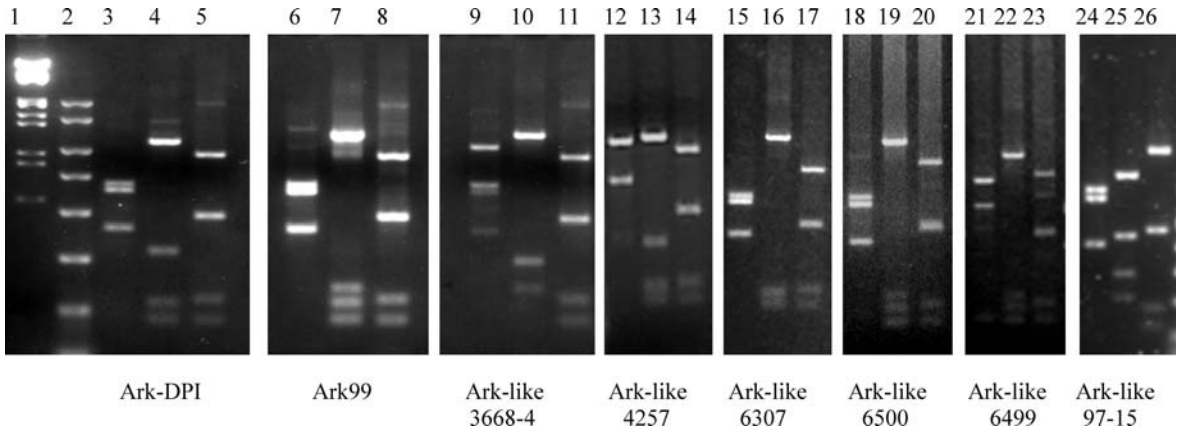

Fig. 4. Restriction fragment length polymorphism patterns for Arkansas and Arkansas-like viruses. Lanes 1 and 2, molecular weight markers; lanes 3–5, Ark-DPI; lanes 6–8, Ark99; lanes 9–11, Ark-like isolate 3668-4; lanes 12–14, Ark-like isolate 4257; lanes 15–17, Ark-like isolate 6307; lanes 18–20, Ark-like isolate 6500; lanes 21–23, Ark-like isolate 6499; lanes 24–26, Ark-like isolate 97-15. Lanes 3, 6, 9, 12, 15, 18, 21, and 24 are digested with *Bst*YI; lanes 4, 7, 10, 13, 16, 19, 22, and 25 are digested with *Hae*III; lanes 5, 8, 11, 14, 17, 20, 23, and 26 are digested with *Xcm*I.

variant viruses, were submitted and typed during that time. Many U.S. poultry companies cut back on bronchitis vaccination by administering less than a full dose of vaccine per bird or eliminating one or more serotypes from a multiple-serotype vaccination program during the warmer months of the year. This practice may allow existing variant viruses to proliferate and cause disease in chickens with reduced immunity to IBV. It could also provide an environment for new IBV variants to develop by allowing viruses to cycle in susceptible birds.

The most frequently identified IBV type was the Ark-DPI strain followed by Conn and Mass. The common vaccine types used in the United States are Ark-DPI, Conn, DE072, and Mass. Our data show that vaccine type viruses are the most frequently isolated IBV types, except in years when a new variant virus has emerged. It is not clear why relatively few isolations of DE072 were detected. Unfortunately, a distinction between vaccine and field viruses of the same serotype cannot be made with any typing method available to date. However, the predominance of vaccine type viruses is probably not coincidental and reinforces the recommendation that vaccines should be used responsibly.

The high percentage of Ark-DPI virus isolations each year is intriguing. Perhaps this reflects the amount of Ark vaccine used in the field or that the Ark-DPI types have a selective advantage over other types of IBV in the upper-respiratory tract of chickens, persist for longer periods in the birds, or are more easily isolated. Whatever the explanation, this observation is extremely important because understanding the mechanism that contributes to the predominance of Ark type viruses in the field may lead to improved methods of control. In addition to their predominance in the field, it appears that Ark type viruses are also evolving because new Ark-like types continue to emerge (Fig. 3). Ark-like viruses are defined as virus isolates with an RFLP pattern similar but not identical to Ark-DPI (Fig. 4). It is logical to assume that persistence of Ark in chickens provides an opportunity for that virus to undergo genetic drift. Fortunately, it appears that antigenic drift or shift has not occurred because Ark vaccines were reported to adequately protect against Ark-like viruses (13).

Variant IBV types are continuously evolving in commercial chicken flocks and periodically emerge to cause widespread disease (2,11,12). Variant IBV types, using the RT-PCR/RFLP typing test, are defined as a virus with an RFLP band pattern that is different from known IBV types. This could be a difference in the size or number of bands in the RFLP pattern or both. Variant viruses are always a concern, but only when a variant virus is detected numerous times in a short period of less than 1 yr, as was observed with GAV and GA98, does it generally become widespread and cause significant disease. It is assumed that this occurs because conventional vaccines no longer protect against the variant; however, this needs to be confirmed by *in vivo* testing.

Differences in virulence of IBV types have been reported (3), and ultimately we are most interested in those viruses with the potential to cause disease. Because all of the clinical samples submitted to PDRC were from birds with clinical signs characteristic of IBV, we believe the data are representative of the important IBV types circulating in the field and having the potential to cause disease outbreaks.

Constantly monitoring IBV types has allowed us to follow changes in the incidence and distribution of IBV and to identify and control new problematic IBV types as they arise. Our data indicate that variant IBV types are constantly circulating in the field and that disease outbreaks associated with variant types occur periodically. That information highlights the importance of constantly monitoring variant IBV isolates as well as other coronaviruses, like SARS-CoV, that have the potential to change and emerge to cause disease in a susceptible host (4).

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